RESEARCH PROPOSAL for PhD studies at Symbiosis International University

Dr Usha Agrawal (SIRI ID 2000615)

Proposed Topic of Research

Role of Biologic Response Modifiers in Recurrence of Urothelial Bladder Carcinoma

Rationale and Significance of the Study

Urinary bladder cancer is the 6th most common cancer, surpassed in frequency only by cancers of the lung, larynx, tongue, prostate, and esophagus (Delhi Cancer Registry, 2003). The majority of the patients present with superficial disease and are treated by transurethral resection of the bladder tumor. More than half of these patients experience recurrence, with about 20% progressing to muscle invasive disease. The natural history of bladder cancer is largely unpredictable owing to tumor heterogeneity. Adjuvant chemotherapy and immunotherapy had been used as a prophylactic measure to reduce the frequency of recurrences with variable success rate. The traditional approaches to detect and monitor bladder cancers are not sufficiently predictive in the individual patients and therefore a strong need exists for additional methods to predict bladder cancer behavior and prognosis. Earlier reports have shown that patients having bladder malignancies were immunodeficient manifesting as impaired cell-mediated and humoral immunity as well as by impaired non-specific host defence mechanisms. Hence, control of tumour growth presents a difficult problem for the host immune system. The identification of **defects in host antitumour immunity** responsible for recurrences can help in identifying predictors of tumor behavior as well as targets for immunotherapy. It would also help in understanding the biologic profile responsible both for recurrence and resistance to treatment.

Literature Review

Urothelial/Transitional Cell Carcinoma (UC/TCC) is the commonest cancer of urinary bladder, 70% being superficial at the time of first clinical presentation [1]. Adjuvant chemotherapy and immunotherapy had been used as a prophylactic measure to reduce the frequency of recurrences in Urothelial cancer with variable success rate. The traditional approaches to detect and monitor bladder cancers are not sufficiently predictive in the individual patients and therefore a strong need exists for additional methods to predict bladder cancer behavior and prognosis. In superficial TCC of urinary bladder, intravesical drug treatment is a modality that provides direct delivery of the drug to the tumor area, avoiding factors controlling systemic absorption and delivery of drug to the tumor area, and also reducing systemic toxicity. Role of *in vitro* cytotoxicity assessment and therapy based on its results has been studied in superficial TCC in order to give individualised treatment and tailoring of dose for various cytotoxic/immunomodulating agents which has helped in reducing recurrence to 14.8% compared to 37.5% in bladder cancer patients and also

showed BCG as potent immunomodulator reducing the recurrence significantly when given following *in vitro* cytotoxicity assessment [2]. Failure of response to therapy even after modifying treatment based on results of repeat *in vitro* cytotoxicity assessment had been explained due to inability of the host to mount an adequate immune response or presence of resistant tumor clones derived from different progenitor cells.

Bladder malignancies have been shown to be immunodeficient [3]. Immune cells infiltrate tumors and make up a significant component of the multicellular cancer micro-environment, yet the immune system often fails to prevent tumor formation and progression. One explanation for this paradox is the presence of tolerance-promoting regulatory T cells (Tregs) that counteract antitumor immune cells. Moreover, Treg infiltration in human tumors and malignant ascites is associated with worse clinical outcomes for various types of cancers [4]. However, the role of immunological dysfunction in progression and recurrence of superficial bladder cancer has not been well elucidated.

Cytokines are a unique family of growth factors. Secreted primarily from leukocytes, cytokines stimulate both the humoral and cellular immune responses, as well as the activation of phagocytic cells. According to the cytokine profile, the immune responses have been classified into 2 functionally distinct CD4+ T-cell subsets. The cytokines produced by the Th1 and Th2 cell subsets are important for the function and immune response of cytotoxic T-lymphocytes to regulate the differentiation of these cells. The characteristic cytokine products of Th1 and Th2 cells are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype. This cross regulation may partly explain the strong biases towards either Th1 or Th2 responses associated with many pathological conditions [5].

Very few studies of the Th1 and Th2 balance have been performed in cancer patients [6, 7] moreover the role of two subsets in anti-tumor immunity in bladder cancer patients is also not clearly defined. The predominant expression of Th1 cytokines is important for anti-tumor immunity [8] and IL-2 has been found to inhibit the invasive and metastatic potential of hepatocellular carcinoma cells. Increased serum levels of IL-10 have been associated with poor prognosis in patients with solid tumors. Th1- and Th2-type cytokines normally maintain a balance. In recent years, it has been revealed that Th2-type cytokines are expressed predominantly in patients with cancers [9].

The ability to assess cytokine profile in different immune cells is fundamental to understand both normal immunoregulation and its dysfunction in Urothelial bladder cancer. Profiles of cytokine reactivity have been extremely important determinants of disease outcome for a large number of infections. It has been made clear by many investigators that production of most cytokines is not confined to one cell type. Thus,

a method to detect cytokines at the single cell level would be helpful to study the contribution of different cell types to cytokine production in heterogeneous cell populations in bladder cancer patients.

It has been observed that cellular level cytokine assessment has been problematic, relying on *in situ* hybridization, limiting dilution, plaque/ ELISPOT or T-cell cloning techniques [10]. All these approaches are laborious and complex, and have practical difficulties. Investigators have usually measured plasma or culture supernatant cytokine titres, which reflect the combinations of many cells and / or a physiological environment. Moreover, this type of analysis does not measure cytokine profile at single cell level. Cytokine expression in T-cells has also been analyzed by RT-PCR, but the limitation of the type of analysis is that it does not give information about the contribution of different cells to cytokine production in a heterogeneous cell population. With technical advancement it has now become possible to investigate cytokine/chemokine profile on a cellular level by cDNA microarray as well as detection of soluble cytokine proteins present in serum/urine by using cytokine bead array mutiparameter flow cytometry or by the xMAP (luminex) technology.

Though no single marker is able to accurately predict the clinical course of bladder tumors and serve as a reliable indicator for recurrence, a combination would predict which superficial tumors need an adjuvant immunotherapy or aggressive form of chemotherapy. So far, the search for target proteins has been done by a candidate gene approach which has shown usefulness of one or two markers at a time. It is pertinent now to study the expression of a large number of genes in tumour tissue by using high-throughput techniques like microarray which would help not only in identifying predictor/transforming proteins for tumour recurrence and resistance to treatment. In addition, their interaction together should be evaluated to understand the molecular mechanism responsible for recurrences. Association of host immunologic profile with progression or recurrence would help in identifying targets for immunotherapy and understanding of the biology of this tumour.

Aims and Objectives of the Study

To study the role of biologic response modifiers in tumour characteristics such as recurrence and progression in Urothelial Bladder Carcinoma

1. To study the expression profile of cytokine/chemokine genes in tumor and surrounding normal tissue and correlation with tumor characteristics.
2. To evaluate expression profile of urinary cytokines in bladder cancer patients with correlation to clinical characteristics and expression profile of cytokine genes in tumour/normal tissue.
3. To evaluate expression profile of serum cytokines in bladder cancer patients with correlation to urinary cytokines.

Scope of the study

Control of tumour growth presents a difficult problem for the host immune system. The exact mechanisms of tumor clearance/recurrence in patients with superficial TCC have not been clearly delineated. The identification of the **transforming proteins,** which transform malignant cells resistant to immuno/chemotherapy as well as **defects in host antitumour immunity** responsible for recurrences can help further in identifying predictors of tumor behavior as well as targets for immunotherapy. It would also help in understanding the biologic profile responsible both for recurrence and resistance to treatment.

The mixture of cytokines that is produced in the tumour microenvironment has an important role in cancer pathogenesis. Cytokines that are released in response to infection, inflammation and immunity can function to inhibit tumour development and progression. Alternatively, cancer cells can respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis. A more detailed understanding of cytokine–tumour-cell interactions provides new opportunities for improving cancer immunotherapy. This study is aimed at the simultaneous evaluation of local and systemic immune alterations in response to the inflammatory pathways. This will in turn help in modulating therapeutic modalities which at present aim at a local immunomodulation to prevent recurrence.

Research Methodology

This study will include 50-60 patients of bladder cancer presenting to the Outpatient Department of Urology, Safdarjang Hospital, New Delhi over a period of two years. Patients will be diagnosed by cystoscopy, undergo transurethral resection of bladder tumor (TURBT), followed by histopathological examination.

Gene Expression profile by Superarray

(A) Total RNA Isolation: Total RNA will be extracted from frozen tumour and matched normal tissue by using RNA isolation kit from Qiagen and following the protocol of the manufacturer. It will be further treated with DNAse to remove any residual contamination with genomic DNA.

(**B**) **Qualitative and Quantitative estimation of RNA:** RNA integrity will be checked by 2100 Bioanalyser (Agilent, Palo Alto, CA). RNA quality will be determined by Nanodrop® ND-1000 UV-V Spectrophotometer (Nanodrop Technology, Rockland, USA)

(D) PCR array and Image Analysis: Genomic DNA contamination will be eliminated by Dnase treatment by using RNAse free DNAse kit (Qiagen GmbH, Hilden, Germany). Superarrays related to NFkB pathway and cytokine Th1-Th2-Th3 profile will be procured from

SuperArray Bioscience Corporation (Frederick, MD). PCR will be performed on ABI Prism 7000 Sequence Detector (Applied Biosystems). For data analysis the ΔΔCt method will be used; for each gene fold-changes will be calculated as difference in gene expression between normal and tumor. A positive value indicates gene up-regulation and a negative value indicates gene down-regulation.

Immunologic parameters

To study the host immune mechanism responsible for antitumor action of immune competent cells in tumour tissue by cytokine array.

Collection of blood: 10 ml blood will be collected from bladder cancer patients and normal healthy volunteers and processed within1 hr of withdrawal. Blood samples will be centrifuged at 3500 rpm at RT and stored frozen in aliquots at –70°C until use.

Collection of Urine: 10 ml urine will be collected from bladder cancer patients and normal healthy volunteers. Samples will be filtered and filtrate will be stored frozen in aliquots at –70°C until use.

Th1/Th2 cytokine evaluation and analysis: Cytokine analysis in serum and urine will be performed by multiplex assay for a panel of 27 cytokines, chemokines and growth factors (Bioplex, Biorad). During analysis, the mean fluorescence intensity (MFI) value for the PE-associated fluorescence will be recorded for each population of cytokine capture-beads.

Statistical Analysis

Statistical Analysis of the data would be done by appropriate parametric and non-parametric methods of analysis.

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